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AN EFFICIENT *IN VITRO* PROPAGATION PROTOCOL OF *DIANTHUS GIGANTEIFORMIS* BORBAS SUBSP. *KLADOVANUS* (DEGEN) SOO

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Abstract: *Dianthus giganteiformis* subsp. *kladovanus* is an endemic, endangered, horticulturally appealing perennial plant that can be used for the revegetation of sand dunes of the Danube region. The appropriate method for its effective production is micropropagation. For this reason, the experiments were conducted in order to establish an efficient protocol for the micropropagation of this subspecies. The sterile culture was initiated from seeds collected *in situ* and the germination percentage was high (88%). According to the results obtained in this study, the multiplication phase should be performed on an MS medium enriched with 0.1 mg/L BAP and 0.1 mg/L NAA. The concentration of MS salts significantly influenced rooting, and higher rooting percentages were obtained on reduced MS media (91.7 - 95%) than on MS media (73.4 - 76.7%). The addition of NAA slightly increased rooting percentage (up to 95%). Obtained microplants were successfully acclimatized (83.3%) in a substrate composed of peat and sand (1: 1; v/v). Using the protocol presented in this paper, the efficient propagation of *D. giganteiformis* spp. *kladovanus* can be achieved for rapid plant production aimed at revegetation, biodiversity protection and floricultural production of this species.

Keywords: *Dianthus giganteiformis* ssp. *kladovanus, in vitro*, plant biotechnology, endangered species

INTRODUCTION

The large number of valuable protected sands, sand steppes and loess steppes are located in the Danube basin and they are highly sensitive to degradation (Jean-Vasile *et al.,* 2013; Biserkov *et al.,* 2015; Kadović *et al*., 2016). The protection or restoration of natural sands vegetation is needed to maintain sandy-steppes and their associated species richnes (Biserkov *et al.,* 2015; Edthofer & Samec, 2016). In Serbia, Kladovo sands is an important habitat, designated as an ecologically important area within the ecological network of the Republic of Serbia. The main negative impacts are surrounding forest plantations, transformation of natural habitats into agricultural lands, spreading of invasive species, grazing and other human activities (Edthofer & Samec, 2016). The conservation management of these areas includes active management to stop spreading of the invasive species and expansion of forest vegetation, followed by the restoration of natural vegetation and *ex-situ* protection of psammophytic endangered species (Šefferová Stanová *et al.,* 2008; Biserkov *et al.,* 2015; Edthofer & Samec, 2016).

Dianthus giganteiformis Borbas subsp. *kladovanus* (Degen) Soo (syn. *Dianthus pontederae* A. Kern. subsp. *kladovanus* (Degen) Stoj. & Stef.) (Jalas & Suominen, 1988: Ciocârlan, 2009) is an endemic subspecies of the Balkan Peninsula. In Serbia, it has the status of a critically endangered (CR) species (Diklić *et al.,* 1999), and is covered by legal protection (Law on Environmental Protection, Rulebook on protected species, 2010). In the neighbouring countries Bulgaria and Romania, it has the status of a rare species, while in Bulgaria it is also protected by law (Petrova, 1997; Oprea, 2005; Petrova & Vladimirov, 2009). In Serbia, this species is found only in the victinity of the village of Davidovac, in Kladovska Sands, where its subpopulation is estimated at 250 individuals with a decreasing tendency (Jalas & Suominen, 1988; Diklić *et al.,* 1999).

D. giganteiformis subsp. *kladovanus* is a perennial psammophytic species which grows in sand steppes and can be used as an ornamental on sandy soils. For this reason, Marković *et al.* (2006) conducted a preliminary research investigating the possiblity of micropropagation of this taxon. However, their research included a small number of treatments only on a half-strength MS medium (Murashige & Skoog, 1962) containing only a single concentration of BAP (1 mg/L), while the multiplication index was low. Since micropropagation is an important method for *ex situ* and *in situ* conservation of endangered taxa (Fay, 1992; Pence, 1999), numerous research papers, investigating the possibilities of successful micropropagation of the endangered or endemic *Dianthus* spp. in the Balkans and Romania, have been published (Cristea, 2010; Jarda *et al.,* 2011; Cristea *et al.,* 2013a, 2013b, 2014; Tsoktouridis *et al.* 2013; Jarda *et al.,* 2014; Marković *et al.*, 2016). Micropropagation protocols obtained in those studies for more than 20 *Dianthus* taxa differ depending on the species or even a genotype of the same species. In order to establish a reliable and efficient protocol for the rapid micropropagation of *D. giganteiformis* subsp. *kladovanus*, we conducted a more detailed investigation of the effect of different media compositions on the multiplication and rooting of shoots on MS and half-strength MS media. In this way, not only its *in situ* and *ex situ* conservation will be enabled but also the production of a large number of plants necessary for the restoration of sustainable natural habitats.

MATERIAL AND METHOD

The seeds were randomly collected from different plants in Kladovska Sands, brought to the laboratory and used for initiating the *in vitro* culture. The 4% NaOCl solution was used for the seeds surface disinfection, followed by rinsing three times in sterile distilled water. The seeds were placed to germinate in full day light conditions (16 h light / 8 h dark). In all experiments the MS medium or half-strength MS medium (Murashige & Skoog, 1962) with the addition of 3% (w/v) sucrose, 0.8% (w/v) agar and different concentrations of plant growth regulators were used. Germination was performed on a hormone-free MS medium.

At the multiplication stage, plant growth regulators Benzylaminopurine - BAP (0.1, 0.5 or 1.0 mg/L) and Naphthaleneacetic acid - NAA (0.1 or 0.5 mg/L) were added to the media (MS or half-strength MS). Three types of explants were used, including single-node cuttings (with 2 axillary buds), terminal buds (containing only apical bud and small part of stem bellow) and terminal shoot cuttings with one node (containing apical bud and one pair of axillary buds). The explants were incubated at 24 ± 2°C, under long day conditions (16/8 h photoperiod), under a light intensity of 50 µmol/m^2s, for 25 days subculturing intervals. Each treatment was repeated three times with 20 explants of the same type. The following parameters were recorded: the number of shoots and nodes developed per each explant and shoot length. In order to avoid a high variability of data for a shoot length, the shoots were grouped into three categories (shorter than 10 mm, 10-20 mm and longer than 20 mm), and the number of shoots in each length category was expressed as a percentage of the total number of shoots.

The rooting of *in vitro* obtained microshoots (10-35 mm long) was performed on the media supplemented with NAA (0.05, 0.1, 0.5 mg/L) or without growth regultors. The experiments were repeated 3 times with 30 explants per treatment. The rooting percentage, the number of roots per microplant, and length of the longest root were determined after 15 days in culture. The uniformly rooted plantlets were treated with a fungicide (1.5% solution of Previcur-N) and acclimatized in a 1: 1 mixture of peat and sand for 25 days before

their survival rate was recorded. In order to maintain high relative humidity, during the first 15 days the microplants were covered with a transparent plastic.

The obtained data were subjected to statistical analysis using the program Statgraphics, version 5.0 (STSC Inc. USA). The percentage data were arcsine-transformed before statistical analysis. The analysis of variance (ANOVA, $p < 0.05$) and the method of least significant difference (LSD) were performed to determine the differences between the treatments.

RESULTS AND DISCUSSION

The seeds successfully germinated in the sterile culture with a high germination percentage (88%), which is an important factor for preserving a population variability after propagation. Although this value is lower than the germination rate achieved by *D. pinifolius* - 92% or *D. carthusianorum* - 95% (Marković *et al.* 2016; Muszyńska & Hanus-Fajerska, 2017), it is significantly higher than the germination percentage *in vitro* of some other *Dianthus* species, such as *D. glacialis* - 31%, *D. giganteus* ssp. *croaticus* - 42%, *D. ingoldbyi* - 65%, or *D. henteri* - 75% (Colombo *et al.*, 2004; Radojević *et al.,* 2010; Pop & Pamfil, 2011; Arda *et al.,* 2016). According to the ISTA (International Rules for Seed Testing) rules for some commercially important *Dianthus* species, the pre-chilling treatment is recommended for dormancy breaking (ISTA, 2011). However, in our research the seeds were sown immediately after collection, without the cold pre-sowing treatment. Perhaps this could be an explanation for the low germination percentage obtained with *Dianthus* spp. in the above mentioned studies. In addition, some authors found that a germination of certain *Dianthus* species can be considerably better in light than in a dark conditions (Marcu *et al.,* 2006), although there were some other reports indicating that light conditions had no impact on germination of some *Dianthus* taxa (Kolodziejek *et al*., 2018). Similarly, there is no recommendation for conducting *Dianthus* germination in the light in the ISTA rules (ISTA, 2011). Besides light, the sterilizing agent and treatment duration can also affect germination rate (Miyoshi & Mii 1995; 1998; Lee *et al.,* 2007), and thus the low germination rate of *D. giganteus* ssp. *croaticus* can be a result of the low NaOCl (1%) concentrations used for disinfection (Radojević *et al.,* 2010). Similarly, the germination of *D. callizonus* was much higher *in vitro* (80%) than *ex vitro* (46%) (Catana *et al.,* 2013). In some cases, gibberellic acid (GA_3) added in growing medium, as an antagonist of ABA (Abscisic acid) which inhibited germination, positively influenced germination (Watkinson & Pill, 1998). For example, the germination rate of *D. henteri* was 100% on the medium with 100 mg/L GA_3, while it was only 75% on the same medium without GA_3 (Cristea *et al.,* 2010).

The percentage of shoot regeneration at the multiplication stage was high, reaching over 93% in the majority of the media tested (Table 1). The concentration of MS salts (MS or half-strength MS) did not affect the frequency of shoot regeneration. On the other hand, there were some small differences in the percentage of regeneration depending on the explant type, since higher regeneration

Table 1. Shoot regeneration on MS and half strength MS (1/2 MS) media

BAP mg/L	NAA mg/L	single node cuttings (%)		terminal buds (%)		shoot cuttings (%)	
		1/2MS	MS	1/2MS	MS	1/2MS	MS
1.0	0.5	$83,3^{bc}$	$91,7^{ab}$	$100,0^{a}$	$90,0^{abc}$	$96,7^{ab}$	$93,3^{b}$
1.0	0.1	$91,7^{ab}$	$93,3^{ab}$	$93,3^{ab}$	$93,3^{ab}$	$95,0^{ab}$	$95,0^{b}$
0.5	0.5	$93,3^{ab}$	$93,3^{ab}$	$98,3^{a}$	$96,7^{ab}$	$100,0^{a}$	$100,0^{a}$
0.5	0.1	$93,3^{ab}$	$96,7^{a}$	$100,0^{a}$	$100,0^{a}$	$98,3^{ab}$	$100,0^{a}$
0.1	0.1	$98,3^{a}$	$98,3^{a}$	$100,0^{a}$	$100,0^{a}$	$100,0^{a}$	$100,0^{a}$

Note: The values followed by different letters are significantly different according to the LSD test, at the $P < 0.05$ level.

Table 2. The average number of shoots and nodes regenerated on different explants after 25 days of *in vitro* culturing

BAP mg/L	NAA mg/L	single node cuttings		terminal buds		shoot cuttings	
		MS	1/2MS	MS	1/2MS	MS	1/2MS
No. of shoots per explant							
1	1	3,1^{ab}	3,3^{ab}	3,2^{ab}	3,8^{ab}	4,1^{a}	3,8^{ab}
1	0,5	3,3^{ab}	3,9^{ab}	3,4^{ab}	2,6^{ab}	3,6^{ab}	3,8^{ab}
0,5	0,5	2,9^{ab}	4,4^{a}	4,6^{a}	2,8^{ab}	4,0^{a}	3,9^{ab}
0,5	0,1	3,7^{a}	4,5^{a}	4,1^{ab}	4,2^{a}	3,7^{ab}	4,1^{ab}
0,1	0,1	3,6^{a}	2,7^{ab}	2,8^{bc}	1,7^{abc}	3,7^{ab}	3,5^{bc}
No. of nodes per explant							
1	1	4,1^{c}	7,5^{ab}	7,2^{ab}	11,4^{a}	10,5^{ab}	8,2^{ab}
1	0,5	6,3^{ab}	9,4^{a}	8,8^{a}	7,5^{b}	6,0^{b}	7,2^{b}
0,5	0,5	9,2^{a}	9,1^{a}	5,1^{b}	8,2^{b}	9,8^{ab}	8,4^{ab}
0,5	0,1	3,7^{c}	4,5^{c}	4,9^{b}	7,1^{b}	9,3^{ab}	9,4^{a}
0,1	0,1	8,5^{ab}	7,9^{ab}	6,5^{ab}	4,7^{c}	12,5^{a}	11,8^{a}

Note: The values followed by different letters are significantly different according to the LSD test, at the P < 0.05 level.

rates were achieved with shoot cuttings compared to terminal buds and single-node cuttings. A similar result was reported for *D. serotinus,* whose regeneration rate of shoot cuttings was higher than the regeneration rate of single node cuttings, whereas the concentration of MS salts significantly affected the shoot regeneration of *D. serotinus*, showing better results on half-strength MS media (Marković *et al.,* 2013).

The mean number of shoots regenerated per explant ranged between 2.6 and 4.6 (Table 2), but a statistically significant impact of a hormone or MS salt concentration on the number of shoots could not be observed. The mean number of shoots can be significantly different depending on the auxine type. Thus, during the micropropagation of *D. carthusianorum,* the number of shoots was only 1.7 on MS medium supplemented with 1 mg/L BAP and 0.2 mg/L NAA, but it was 8 times higher on the same medium with 0.2 mg/L IAA (Indole-3-acetic acid) instead of NAA (Muszyńska & Hanus-Fajerska, 2017). However, the mean number of nodes (Table 2) was considerably higher than in the preliminary research of this subspecies micropropagation (Marković *et al.,* 2006). While the average number of nodes obtained in the research conducted by Marković *et al.* (2006) ranged between 3.5 and 7.6, on 50% of the media tested in this research, the average number of nodes exceeded 7.6, reaching 12.5 nodes regenerated per explant (Table 2).

Generally, the shoots were longer on media with lower concentrations of plant growth regulators, and more than 30% of them were longer than 20 mm on media with 0.1 mg/L BAP and 0.1 mg/L NAA, for all explant types (Figure 1). The impact of the concentration of MS salts is not significant, but generally longer shoots develop on MS media. The impact of the concentration of MS salts on shoot length was recorded for *D. serotinus and D. pinifolius (*Marković *et al.,* 2013, 2016*), with longer shoots developed on full strength MS media.* Nevertheless, half strength MS media can have a favourable effect on shoot regeneration (Desilets *et al.* 1993; Daud *et al.* 2011; Marković *et al.* 2013).

Rooting on half strength MS media was successful, as the rooting percentage ranged from 91.7 - 95%, which corresponds with the preliminary results obtained by Marković *et al.* (2006), who recorded a rooting percentage of 94% on a hormone free half-strength MS medium. Contrary

Figure 1. The effect of different BAP and NAA concentrations added to MS and half-strength MS media on the length of *D. giganteiformis* spp. *kladovanus* shoots regenerated on single node cuttings (A), terminal buds (B), shoot cuttings (C)

Table 3. Rooting of shoots after 15 days of *in vitro* culturing

NAA mg/L	MS salts	Rooting percentage (%)	No. of roots per explant	Mean length of the longest root (mm)
0.0	MS	73,4[b]	3,2[b]	17,5[ab]
0.05		73,4[b]	3,1[b]	16,2[ab]
0.1		75,0[b]	3,8[b]	15,1[ab]
0.5		76,7[b]	4,6[b]	18,4[a]
0.0	1/2MS	93,4[a]	8,9[a]	22,5[a]
0.05		91,7[a]	9,1[a]	18,5[a]
0.1		91,7[a]	9,6[a]	17,4[ab]
0.5		95,0[a]	10,2[a]	19,0[a]

Note: The values followed by different letters are significantly different according to the LSD test, at the P < 0.05 level.

to expectations, NAA concentration neither had an impact on the rooting rate nor on the number of roots per explant (Table 3). The addition of auxine to the medium generally promotes rooting, and sometimes high concentrations of auxines are necessary for the rooting of some *Dianthus* taxa (Salehi, 2006; Papafotiou & Stragas, 2009) or for a more efficient rooting (Tsoktouridis *et al.* 2013). On the other hand, there were also different reports on *Dianthus* spp. micropropagation, in which rooting was successful on a hormone-free medium, including *D. ciliatus* ssp. *dalmaticus*, *D. mainensis* and *D. spiculifolius* (Radojević *et al.,* 2010; Cristea *et al.*, 2013b; Erst *et al.* 2014).

The concentration of MS salts significantly influenced rooting, and the percentage was considerably higher on half strength MS media (table 3), which was also obtained for *D. mainensis* (Erst *et al.* 2014). However, during the micropropagation of *D. pinifolius,* the rooting percentage was higher on MS media than on half-strength MS media (Marković *et al.* 2016), and, in some cases (e.g. *D. nardiformis*), the concentration of MS salts (MS, 1/2MS or 1/4MS) had no significant effect on the percentage of rooting (Holobiuc *et al.* 2010). The addition of NAA did not influence the mean number of roots and root length (Table 3), like in the case of *D. pinifolius* (Marković *et al.* 2016) or *D. gratianopolitanus* (Fraga *et al.* 2004). On the other hand, the auxine type can considerably influence the rooting rate (Marcu *et al.* 2006).

The acclimatization rate obtained in this study is 83.3%, which can be considered satisfactory. Similar results were reported for other *Dianthus* taxa, including *D. mainensis* - 83%, *D. trifasciculatus* ssp. *parviflorus* - 85% and *D. pinifolius* - 88.9% (Holobiuc *et al.* 2013; Erst *et al.* 2014; Marković *et al.* 2016). A lower acclimatization percentage was obtained for *D. fruticosus* - 70% (Papafotiou & Stragas, 2009) but in some cases a higher acclimatization rate was achieved, e.g. by *D. petraeus*, 90-100% (Tsoktouridis *et al.* 2013) or *D. caryophyllus* cultivars - 90% (Salehi, 2006).

CONCLUSIONS

The endangered and decorative subspecies *D. giganteiformis* spp. *kladovanus* can be successfully propagated using the protocol presented in this research. The *in vitro* culture was established from seed collected from different plants in the same population, so the obtained results present the average response to the culture conditions. Although the multiplication phase should be performed on an MS medium containing 0.1 mg/L BAP and 0.1 mg/L NAA, *in vitro* rooting should be on a half-strength MS medium, while the addition of 0.5 mg/L NAA can have a favourable effect. Further, the rooted microplants can be successfully acclimatized in a 1:1 mixture of peat and sand. In this way, efficient *D. giganteiformis* spp. *kladovanus* propagation can be achieved for both plant production aimed at biodiversity protection and the floricultural production of this species.

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